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Aph-2/Nicastrin: An Essential Component of γ-Secretase and Regulator of Notch Signaling and Presenilin Localization

Minireview

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The Notch signaling pathway plays a role in cell fate specification in many metazoans. A critical aspect of Notch activation involves proteolysis of the Notch receptor. This cleavage event requires Presenilin as a component of a large multiprotein complex, γ-secretase. This complex mediates a similar cleavage event of the β-amyloid precursor protein (APP). The transmembrane protein Nicastrin has been found to associate with Presenilin, Notch, and APP. Recent biochemical and genetic studies have focused on elucidating the function of this protein.

The Notch family of proteins encode a family of large type I transmembrane receptors mediating short-range signals elicited by binding to type I transmembrane ligands, known collectively as DSL proteins (*Delta*, *Serrate*, and *Lag 2*). In many metazoans, notch-mediated signals permit equivalent cells to acquire the proper fate, both during development and in the adult. Ligand binding facilitates shedding of the Notch extracellular domain by a metalloprotease, creating a substrate (NEXT, Notch extracellular domain truncated) for intramembranous proteolysis that releases the Notch intracellular domain (NICD). NICD release is mediated by an enzymatic complex called γ secretase, a founding member of a rapidly growing family of intramembrane-cleaving proteases (or I-CliP's). Cleavage of all four mammalian Notch proteins (Notch 1–4) occurs at a specific, conserved position (Saxena et al., 2001). NICD contains a nuclear localization signal, and once released it enters the nucleus where it interacts with a CSL protein (*CBF, Su(H)*, and *Lag-1*) and modifies transcription of a specific set of genes.

Mutations in presenilin-1 (PS1) and presenilin-2 (PS2) are the most common known cause of autosomal dominant familial Alzheimer's disease (FAD). FAD mutations affect the metabolism of the β-amyloid precursor protein (APP), a type I transmembrane protein and the precursor of the Aβ peptides that aggregate in senile plaques in Alzheimer's disease. The N terminus of Aβ is generated when the APP ectodomain is released by BACE (β-secretase), producing a 99 amino acid membrane-associated C-terminal fragment (C99) with a short extracellular stalk. C99, like NEXT, is a substrate for γ-secretase, which generates the C terminus of Aβ by cleaving at one of several positions within the transmembrane domain of C99. Unlike the site-specific cleavage of Notch, γ-secretase cleavage of C99 also generates shorter or longer Aβ species (39–43 residues) in addition to the predominant 40 amino acid long (Aβ 40) Aβ peptide. Like NICD, the carboxyl-terminal product of this cleavage event is translocated to the nucleus (Cao and Sudhof, 2001; Cupers et al., 2001; Gao and Pimplikar, 2001; Kimberly et al., 2001). However, the functional significance of this translocation is presently unknown.

γ-secretase, therefore, plays a critical role in normal processes as well as in the progression of a devastating adult onset disease. Biochemical purification of γ-secretase activity revealed that it is present in a high molecular weight fraction (Li et al., 2000a, 2000b). Presenilin is an obligate component of this enzyme. Several recent publications suggest that one additional, essential component of γ-secretase is the protein Aph-2/Nicastrin.

The Identification of Aph-2/Nicastrin: A Role in Notch Signaling and in γ-Secretase

In *C. elegans*, signaling via Glp-1, the Notch ortholog, first acts early in development to instruct two anterior blastomeres (ABa and ABp) to assume different developmental fates and later plays a role in inducing pharynx fates in the descendents of ABa. In recent genetic screens for novel components of Notch signaling, two *aph* genes (anterior-pharynx defective) were identified that were required for both the 4-cell interaction and for induction of anterior pharynx. *aph-1* encodes a novel, conserved poly-topic membrane protein (Goutte et al., 2002). Cloning of aph-2 identified a type I transmembrane protein that accumulates at the cell surfaces of blastomeres expressing either Glp-1 or its ligand. In vitro mixing experiments of the interacting blastomeres suggested that Aph-2(+) could be provided by either type of blastomere for Notch signaling. A subsequent study (Levitan et al., 2001) detected a genetic interaction between *aph-2* and *sel-12*, the worm presenilin, and Lin-12, the other Notch ortholog. Since no genetic interactions were observed when NICD was expressed in *aph-2* mutants, the investigators concluded that, like presenilin, aph-2 acts upstream of NICD production. These studies together indicate that aph-2 is an essential component of many Notch-mediated interactions, but did not detect a requirement for aph-2 in all such interactions (Levitan et al., 2001).

Independently, the vertebrate *aph-2* ortholog was identified as a stoichiometric component of presenilin immunoprecipitates. The protein was named Nicastrin, after the Italian village Nicastro, home to an extended FAD kindred (Yu et al., 2000). Overall, Aph-2 and Nicastrin exhibit 41% homology, and human nicastrin can partially rescue *aph-2* mutations in *C. elegans*, confirming that the two proteins are functionally related. Higher homology is found in a 31 amino acid region in the extracellular domain containing the DYIGS sequence. Nicastrin mutants containing missense mutations or deletions of this sequence modulate Aβ secretion in mammalian cells.

Biochemical analysis of APP proteolysis was performed in cultured wild type mammalian cells overexpressing Nicastrin revealed that Nicastrin-presenilin interactions were insensitive to presenilin mutations, including D385A, which abolishes all γ-secretase activity. Nicastrin also interacts

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with the APP substrates of γ-secretase (APP, C99, and C83). However, these interactions were affected by mutations in both presenilin and Nicastrin. Less C99/C83 was recovered by Nicastrin immunoprecipitation if presenilin^{D385A} was expressed; more C99/C83 was recovered if presenilin containing a FAD mutation was coexpressed (Yu et al., 2000).

Distant similarities between Nicastrin and the aminopeptidase/transferrin receptor superfamily led to the suggestion that the DYIGS motif and surrounding structural elements act as a protein-protein interaction domain. Deletion of the region containing the DYIGS motif results in a reduction in both secretion of Aβ peptides and Nicastrin-presenilin interactions. In contrast, DYIGS mutations resulted in a significant increase in Aβ40/Aβ42 secretion and increased Nicastrin-presenilin interactions. Interestingly, DYIGS mutations in human Nicastrin have some rescuing activity in *C. elegans*, indicating that not all functions of Nicastrin require this motif (Levitan et al., 2001). The DYIGS domain may mediate interactions with presenilin but not its substrates; interaction with APP and Notch were unaffected by removal of this domain.

Mutations in APP that increase β-secretase cleavage (e.g., APP^{swe} "the swedish mutation") and, thus, production of Aβ peptides by γ-secretase, are suppressed by overexpressing either PS1^{D385A} or Nicastrin$^{\Delta 312\text{-}369}$. However, in contrast to PS1^{D385A} mutants, the reduction in Aβ secretion induced by Nicastrin$^{\Delta 312\text{-}369}$ was not accompanied by an accumulation of APP substrates (C99/C83) in immunoprecipitates from transfected cells. It was concluded that APP stubs that do not bind to the PS/Nicastrin complex may be degraded rapidly by other pathways. However, such degradation is not observed when a dominant-negative presenilin is expressed in the same cell type. Nicastrin$^{\Delta 312\text{-}369}$ could act as a dominant-negative protein; in cells containing wild-type Nicastrin, it reduced Aβ secretion. Alternatively, it is possible that Nicastrin$^{\Delta 312\text{-}369}$ only alters the *trafficking* or *secretion* of Aβ but not APP proteolysis. This possibility was left unresolved since only secreted Aβ was assayed, not total Aβ. Like presenilin, Nicastrin also binds to Notch lacking the extracellular domain (γ-secretase substrate) but not to NICD (Chen et al., 2001; Ray et al., 1999). Nicastrin mutations appear to affect Notch proteolysis to a lesser extent than APP proteolysis. Like their effect on Aβ secretion, Nicastrin$^{\Delta 312\text{-}369}$ reduced and Nicastrin$^{DYIGS\rightarrow AAIGS}$ increased cellular NICD production by a negligible amount.

Collectively, these biochemical observations suggest that an intimate link exists between Nicastrin, APP, presenilin, and γ-secretase activity, but they do not offer a clear mechanistic role for Nicastrin. A role for Nicastrin as a regulatory or catalytic component of γ-secretase has been suggested. Since presenilin-substrate interactions have only been tested in the presence of Nicastrin, it is possible that Nicastrin is the substrate presenting component of γ-secretase. However, this conclusion stands in contrast to the limited role of Aph-2 in Notch signaling, the minimal effect of DYIGS mutants on Notch proteolysis in cultured cells, the ability of Aph-2 to function in the ligand presenting cell, and the observation that Nicastrin$^{\Delta 312\text{-}369}$ could rescue some phenotypes in *aph-2* mutant animals (Levitan et al., 2001). To reconcile these observations, it was proposed (Chen et al., 2001) that perhaps Notch and APP are processed by somewhat different configurations of γ-secretase, one that fully depends on Nicastrin (γ-secretase^{APP}) and the other capable of working without it (γ-secretase^{Notch}). γ-secretase^{Notch} may even be satisfied with Nicastrin being presented by a ligand expressing cell (Goutte et al., 2000). Support for this suggestion came from the observation that Notch does not compete with APP for γ-secretase activity even when Notch expression exceeded APP expression by 240-fold.

Obligate components of γ-secretase should behave like presenilin; in their absence, all γ-secretase activity should be lost. Is Nicastrin required for Notch proteolysis? Do different γ-secretases exist? The ability of γ-secretase to hydrolyze Notch without Nicastrin has as yet only been observed in *C. elegans* where the perdurance of the maternal proteins confounds the interepretation to some extent. Biochemical experiments in cell culture were all done in the presence of Nicastrin protein, with no demonstration that γ-secretase activity was saturated.

Nicastrin Is Essential for Notch Signaling and Is Indistinguishable from Presenilin in Drosophila

Several recent publications reporting the isolation and characterization of Nicastrin loss-of-function alleles in *Drosophila* address some of these questions and propose an unexpected role for Nicastrin (Chung and Struhl, 2001; Hu et al., 2002; Lopez-Schier and Johnston, 2002). Lopez-Schier and Johnston (2002) isolated a lethal complementation group called *agoraphobic* (*agro*) and found it to be identical to Nicastrin. The *agro* mutation created a truncated Nicastrin protein lacking the transmembrane and cytoplasmic domains. Embryos lacking both maternal and zygotic *Nicastrin^{agro}* display a strong neurogenic phenotype, identical to that observed with a loss of Notch signaling. Similar observations are reported by the two other groups which independently isolated alleles of Nicastrin. Each of these alleles results in truncated proteins that are no longer membrane tethered (Chung and Struhl, 2001; Hu et al., 2002).

The function of Nicastrin in Notch signaling was explored in *Nicastrin* null flies (Chung and Struhl, 2001; Lopez-Schier and Johnston, 2002). When homozygous mutant patches of tissue were generated at the wing margin, these clones were identical to clones deficient in Notch signaling. The border between dorsal and ventral territories and the expression of Wingless, a Notch target gene, define the wing margin and form the wing "organizer." Both were lost in *Nicastrin* clones, resulting in a "notched" wing, the classical Notch phenotype. Importantly, the loss of wing margin in *Nicastrin$^{-/-}$* clones is strictly cell-autonomous (Lopez-Schier and Johnston, 2002). Delta-overexpressing *Nicastrin$^{-/-}$* clones in the middle of the wing imaginal disc induced ectopic wing margin in neighboring wild-type cells (Chung and Struhl, 2001), demonstrating that *Nicastrin$^{-/-}$* ligand expressing cells can still activate Notch in their neighbors. Margin cells in which Notch is activated secrete Wingless. Since Wnt signaling is unaffected by Nicastrin (Goutte et al., 2000), both wild-type and *Nicastrin$^{-/-}$* cells respond to Wingless and become competent to acquire the sensory organ precursor (SOP) fate. Wild-type cells use Notch to allow only a few SOP to form, spaced by cells that have lost their competence and become epidermal instead. Many adjacent SOP arose inside the *Nicastrin$^{-/-}$* clone, indicating that Notch-dependent lateral inhibition was

lost. This elegant experiment, and similar ones in the oocyte (Lopez-Schier and Johnston, 2002), demonstrate concomitant cell autonomous loss of Notch signal *reception* in cells competent for signal transmission. This experiment also underscores that β-catenin, which together with α-catenin had previously been shown to coprecipitate with presenilin and Nicastrin, is fully active in Nicastrin-deficient cells (Chung and Struhl, 2001; Goutte et al., 2000).

To establish the precise step during Notch activation where Nicastrin was required, full-length or truncated Notch (extracellular domain-truncated Notch and NICD) proteins were overexpressed in embryos or imaginal disks lacking maternal and zygotic Nicastrin. Only NICD reversed the *Nicastrin* phenotype in these experiments (Chung and Struhl, 2001; Hu et al., 2002; Lopez-Schier and Johnston, 2002).

These observations and the experiments in *C. elegans* position Nicastrin function upstream to NICD and downstream of ligand-induced ectodomain shedding. The accumulation of NEXT fragments in *Nicastrin*^{agro} is reminiscent of the accumulation observed in γ-secretase inhibitor-treated mammalian cells (Mumm et al., 2000). During Notch activation, ligand-mediated ectodomain shedding is accompanied by transendocytosis of the Notch extracellular domain into the ligand-expressing cell. Transendocytosis of the Notch extracellular domain is unaffected in *presenilin*, *Nicastrin*, or *CSL*$^{Su(H)}$ mutants, supporting the notion that Nicastrin only acts after ligand-induced shedding of the extracellular domain to facilitate the intramembrane proteolysis of Notch. Notch trafficking, ligand binding, and subsequent cleavage all occur normally in cells lacking *presenilin* or *Nicastrin*. Biochemical evidence further supports a role for Nicastrin specifically in intramembrane proteolysis of Notch. Hu et al. (2002) found that Nicastrin mutants, presenilin mutants, or pharmacological inhibition of γ-secretase all result in the disappearance of the same Notch fragment, most likely NICD, from cell or embryo extracts. Expression of Notch or APP transmembrane domain fragments fused to the Gal4/Vp16 protein in *Nicastrin*$^{-/-}$ embryos demonstrated that all tethered Gal4/VP16 proteins failed to activate nuclear targets in the absence of Nicastrin protein (Chung and Struhl, 2001).

Nicastrin Is Required for Presenilin Stability

Collectively, these observations demonstrate that, like presenilin, Nicastrin is required for the intramembrane proteolysis of Notch and APP. However, what does Nicastrin contribute to γ-secretase? *Drosophila* again provides a clue. Using RNAi against the 5′ and 3′ untranslated region of *Nicastrin* RNA to create cells depleted of endogenous Nicastrin, Hu et al. (2002) report complete loss of both endogenous and transfected presenilin in RNAi-treated S2 cells with no corresponding change in *presenilin* RNA levels. This indicates a possible role for Nicastrin in posttranslational stabilization of presenilin. Ectopic Nicastrin isoforms bearing the DYIGS mutations were expressed in this system using a heterologous UTR. Nicastrin expression in these RNAi-treated cells results in stabilized presenilin and restored full γ-secretase activity. When mutant forms of Nicastrin protein containing mutations to the DYIGS domain were expressed in these RNAi-treated cells, presenilin was not stabilized and γ-secretase activity was not restored (Chen et al., 2001; Yu et al., 2000).

In vivo, *Nicastrin* was required for presenilin stability as well, but loss of Notch proteolysis was not caused by presenilin instability. In small clones or clones analyzed early in oogenesis, Notch signaling was lost, yet presenilin protein was present at normal levels (Lopez-Schier and Johnston, 2002). Only later in development were presenilin proteins lost in Nicastrin^{agro} clones. If Notch proteolysis is lost while presenilin is present, what is Nicastrin providing?

Nicastrin, Presenilin, *and* aph-1 *Mutants Show Specific Defects in Trafficking*

A clue came from the observation that, in transgenic flies expressing HA-tagged presenilin, anti-HA staining was less intense in *Nicastrin*$^{-/-}$ clones than in surrounding wild-type cells, despite the fact that mitotic recombination also doubles the copy number of HA-presenilin, suggesting that presenilin stability is affected in *Nicastrin*$^{-/-}$ cells (Chung and Struhl, 2001; Lopez-Schier and Johnston, 2002). In addition, Nicastrin appears to be required for apical localization of presenilin. Presenilin accumulates in the ER of Nicastrin-deficient cells. No change in the distribution of Notch was detected in these embryos.

Defects in transport of other proteins were also detected in follicle cells lacking Nicastrin or presenilin but not in cells lacking Notch (Lopez-Schier and Johnston, 2002). For instance, the submembranous organization of the apical spectrin cytoskeleton was subtly altered. No changes were detected in cellular polarity or in the distribution of other presenilin-associated proteins, including Notch, *D*E-cadherin, α- and β-catenin. Basolateral spectrin complexes were also unaffected. Previous experiments in PS1-deficient cells uncovered changes in the trafficking and secretion of APP and TrkB (Naruse et al., 1998). Together, these observations implicate Nicastrin and presenilin in apical transport of some proteins and apical stability of others. Importantly, this apical trafficking activity associated with γ-secretase is not dependent on its proteolytic activity (Beher et al., 2001).

Further evidence coupling transport with the γ-secretase complex comes from *aph-1* mutants, a multipass transmembrane protein with no known function. *aph-1* is essential for multiple Notch-mediated decisions, including regulation of mitosis in the gonad, which is unaffected in *aph-2* mutants. Glp-1 protein distribution in the four-cell stage embryo is unaltered in either *aph-1* or in *hop-1; sel-12* double mutants, which lack all presenilin proteins. Remarkably, Aph-2/Nicastrin apical localization depends on *aph-1* and *hop-1;sel-12* (Goutte et al., 2002).

Conclusions

What then is the most likely function of Nicastrin? How essential is its role? It seems that evidence is mounting that the substrate binding activity of Nicastrin may not be necessary for its function. Loss of presenilin-Nicastrin interactions is correlated with loss of γ-secretase activity, and in *Drosophila*, Nicastrin is independently required for proper localization and stability of presenilin/γ-secretase and its activity (Chung and Struhl, 2001; Hu et al., 2002; Lopez-Schier and Johnston, 2002). While mislocalized presenilin may be rapidly degraded, Nicastrin is required for γ-secretase activity even before presenilin disappears (Hu et al., 2002; Lopez-Schier and

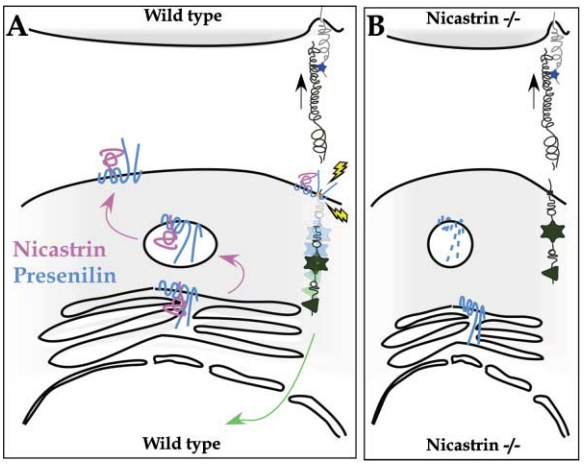

Figure 1. Proposed Role for Nicastrin in Notch Processing and Presenilin Localization and Stabilization

(A) Notch signaling is triggered by ligand binding. Extracellular domain shedding is mediated by a metalloprotease. Presenilin dependent γ-secretase releases the intracellular domain by intramembrane proteolysis. NICD contains six ankyrin repeats (star) and nuclear localization signals (ovals) (see text for details).

(B) In the absence of Nicastrin, extracellular proteolysis occurs normally in response to ligand but Notch signaling is blocked due to failure of preseniln to reach the cell surface.

Johnston, 2002). Since Notch proteolysis occurs within the apical membrane or an associated endosome, mislocalized presenilin could fail to activate Notch. As for APP proteolysis, it too is restricted to a specific vesicular compartment and may require the transport function of Nicastrin to become associated with presenilin. It seems that the best fit for all the available data is that Nicastrin facilitates maturation and exit of presenilin from the ER, where it is inactive (see Figure 1).

If this interpretation is correct, Nicastrin may be an essential component of Notch signaling in all phyla. Loss-of-function experiments have not yet been reported in vertebrates, but the high degree of conservation among proteins involved in Notch signaling makes it likely that Nicastrin is an essential component of all γ-secretase activities, including Notch signaling. Given this, one might predict that Nicastrin would be no better a target for Alzheimer's disease therapeutics than presenilin. As a counter argument, it has been proposed that mutations in, and deletions around, the conserved DYIGS motif could act as dominant modifiers of the Aβ40/Aβ42 ratio with little or no impact on Notch signaling (Chen et al., 2001; Yu et al., 2000). One caveat to this conclusion is that only secretion, not production of Aβ, was measured. The lack of accumulation of C99/C83 stubs might also suggest that APP proteolysis was unaffected. In insect S2 cells lacking Nicastrin, Nicastrin^{DYIGS} fails to restore γ-secretase activity, and no dominant-negative effect is seen when mutants are coexpressed with wild-type Nicastrin (Hu et al., 2002).

Regardless of the mechanism, heterozygous DYIGS mutations in Nicastrin would be expected to present as a viable FAD allele or risk factor for AD in humans since they would affect the secretion of Aβ42 and thus its accumulation. Genetic studies in late onset AD have reported evidence of linkage on chromosome 1 in the region containing the *Nicastrin* gene (Kehoe et al., 1999). To date, sequencing of the *Nicastrin* coding region in individuals with late onset AD has not revealed any polymorphisms or mutations (Yu et al., 2000). However, as Nicastrin is a stoichiometric component of γ-secretase, this analysis should be extended to *Nicastrin* regulatory sequences before its involvement in Alzheimer's disease as a genetic risk factor can be ruled out.

Selected Reading

Beher, D., Wrigley, J.D., Nadin, A., Evin, G., Masters, C.L., Harrison, T., Castro, J.L., and Shearman, M.S. (2001). J. Biol. Chem. *276*, 45394–45402.

Cao, X., and Sudhof, T.C. (2001). Science *293*, 115–120.

Chen, F., Yu, G., Arawaka, S., Nishimura, M., Kawarai, T., Yu, H., Tandon, A., Supala, A., Song, Y.Q., Rogaeva, E., et al. (2001). Nat. Cell Biol. *3*, 751–754.

Chung, H.M., and Struhl, G. (2001). Nat. Cell Biol. *3*, 1129–1132.

Cupers, P., Orlans, I., Craessaerts, K., Annaert, W., and De Strooper, B. (2001). J. Neurochem. *78*, 1168–1178.

Gao, Y.H., and Pimplikar, S.W. (2001). Proc. Natl. Acad. Sci. USA *98*, 14979–14984.

Goutte, C., Hepler, W., Mickey, K.M., and Priess, J.R. (2000). Development *127*, 2481–2492.

Goutte, C., Tsunozaki, M., Hale. V.A., and Priess, J.R. (2002). Proc. Natl. Acad. Sci. USA *99*, 775–779.

Hu, Y., Ye, Y., and Fortini, M.E. (2002). Dev. Cell *2*, 69–78.

Kehoe, P., Wavrant-De Vrieze, F., Crook, R., Wu, W.S., Holmans, P., Fenton, I., Spurlock, G., Norton, N., Williams, H., Williams, N., et al. (1999). Hum. Mol. Genet. *8*, 237–245.

Kimberly, W.T., Zheng, J.B., Guenette, S.Y., and Selkoe, D.J. (2001). J. Biol. Chem. *276*, 40288–40292.

Levitan, D., Yu, G., St George-Hyslop, P.H., and Goutte, C. (2001). Dev. Biol. *240*, 654–666.

Li, Y.M., Lai, M.T., Xu, M., Huang, Q., DiMuzio-Mower, J., Sardana, M.K., Shi, X.P., Yin, K.C., Shafer, J.A., and Gardell, S.J. (2000a). Proc. Natl. Acad. Sci. USA *97*, 6138–6143.

Li, Y.M., Xu, M., Lai, M.T., Huang, Q., Castro, J.L., DiMuzio-Mower, J., Harrison, T., Lellis, C., Nadin, A., Neduvelil, J.G., et al. (2000b). Nature *405*, 689–694.

Lopez-Schier, H., and Johnston, D.S. (2002). Dev. Cell *2*, 79–89.

Mumm, J.S., Schroeter, E.H., Saxena, M.T., Griesemer, A., Tian, X., Pan, D.J., Ray, W.J., and Kopan, R. (2000). Mol. Cell *5*, 197–206.

Naruse, S., Thinakaran, G., Luo, J.J., Kusiak, J.W., Tomita, T., Iwatsubo, T., Qian, X.Z., Ginty, D.D., Price, D.L., Borchelt, D.R., et al. (1998). Neuron *21*, 1213–1221.

Ray, W.J., Yao, M., Mumm, J., Schroeter, E.H., Saftig, P., Wolfe, M., Selkoe, D.J., Kopan, R., and Goate, A.M. (1999). J. Biol. Chem. *274*, 36801–36807.

Saxena, M.T., Schroeter, E.H., Mumm, J.S., and Kopan, R. (2001). J. Biol. Chem., in press.

Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L.L., Tandon, A., Song, Y.Q., Rogaeva, E., Chen, F.S., Kawaral, T., et al. (2000). Nature *407*, 48–54.